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| (51) International Patent Classification ⁶ : C12Q 1/58, C12N 9/12, A01N 25/00 | | A1 | (11) International Publication Number: WO 99/38998 (43) International Publication Date: 5 August 1999 (05.08.99) |
| (21) International Application Number: PCT/US99/02065 (22) International Filing Date: 27 January 1999 (27.01.99) | | (81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> | |
| (30) Priority Data: 60/072,940 29 January 1998 (29.01.98) US 60/077,582 10 March 1998 (10.03.98) US 9807936.1 15 April 1998 (15.04.98) GB 60/085,452 14 May 1998 (14.05.98) US 9823089.9 21 October 1998 (21.10.98) GB 60/106,287 30 October 1998 (30.10.98) US 60/108,306 13 November 1998 (13.11.98) US | | | |
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(54) Title: METHODS OF IDENTIFYING MODULATORS OF KINASES RESPONSIVE TO STRESS**(57) Abstract**

The present invention relates to methods of identifying compounds useful as modulators of certain stress responsive kinases. More particularly, the compounds so identified are useful for treating or preventing diseases or conditions that are mediated by, for example, abnormal bone resorption or angiogenesis. These compounds are useful for treating or preventing osteoporosis, and inhibiting vascular restenosis, diabetic retinopathy, macular degeneration, angiogenesis, atherosclerosis, inflammation, and tumor growth.

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TITLE OF THE INVENTION**METHODS OF IDENTIFYING MODULATORS OF KINASES
RESPONSIVE TO STRESS****5 CROSS-REFERENCE TO RELATED APPLICATIONS**

The present application claims priority of U.S. provisional application Serial No. 60/108,306, filed November 13, 1988; U.S. provisional application Serial No. 60/106,287, filed October 30, 1988; U.S. provisional application Serial No. 60/085,452, filed May 14, 1998; U.S. 10 provisional application Serial No. 60/077,582, filed March 10, 1988; and U.S. provisional application Serial No. 60/072,940, filed January 29, 1998.

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to methods of identifying compounds useful as modulators, particularly activators, of certain stress responsive kinases. More particularly, the compounds so identified are useful for treating or preventing diseases or conditions that are mediated by, for example, abnormal bone resorption or angiogenesis. These compounds are useful for treating or preventing osteoporosis, and inhibiting vascular restenosis, diabetic retinopathy, macular degeneration, angiogenesis, atherosclerosis, inflammation, and tumor growth.

BACKGROUND OF THE INVENTION

It is believed that at least a dozen kinds of kinases responsive to stress exist in various mammalian cells. One of these kinases, the Mammalian Sterile 20-like Kinase has recently been described in the scientific literature. At least two isoforms of Mammalian Sterile 20-like Kinase are known, hereafter referred to as "Mst 1" and "Mst 2". See, Taylor et al., "Newly identified stress-responsive protein kinases, Krs-1 and Krs-2", *Proc. Natl. Acad. Sci. USA*, Vol. 93 (1996), pp. 10099-10104; Creasy et al., "Cloning and characterization of a human protein kinase with homology to Ste20," *The J. of Biological Chemistry*, Vol. 270, No. 37 (1995), pp. 21695-21700; Creasy et al., "Cloning and characterization of a member of the MST

subfamily of Ste20-like kinases", *Gene*, Vol. 167 (1995), pp. 303-306; Creasy et al., "The Ste 20-like protein kinase, Mst 1, dimerizes and contains an inhibitory domain", *The J. of Biological Chemistry*, Vol. 271, No. 35 (1996), pp. 21049-21053; and Wang and Erikson, "Activation of 5 protein serine/threonine kinases p42, p63, and p87 in Rous sarcoma virus-transformed cells: signal transduction/transformation-dependent MBP kinases", *Mol. Biol. Cell*, 3, pp. 1329-1337 (1992), which are all incorporated by reference herein in their entirety. As part of the present invention, three other kinases have been identified using an in-gel 10 kinase assay. These kinases have a molecular weight of approximately 34 kDa, 50 kDa, and 130 kDa and will be referred to herein as "34 kDa Kinase", "50 kDa Kinase", and "130 kDa Kinase", respectively. It is believed that Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase and 130 kDa Kinase play a key role in regulating various mammalian cells such as 15 osteoclasts and in regulating cellular processes such as angiogenesis.

It is believed that a wide variety of disease states and conditions can be mediated by modulating, for example by activating, Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, or 130 kDa Kinase, or combinations thereof. It is also believed that so-called modulators of 20 these kinases represent a new and useful therapeutic class of drugs. Such modulators would be useful for treating or preventing diseases which include osteoporosis, osteopenia, and Paget's disease, and for inhibiting vascular restenosis, diabetic retinopathy, macular degeneration, angiogenesis, atherosclerosis, inflammation, and tumor 25 growth.

In one aspect of the present invention, the activator compounds herein are useful for inhibiting bone resorption. Bone resorption is mediated by the action of cells known as osteoclasts. Osteoclasts are large multinucleated cells of up to about 400 μm in 30 diameter that resorb mineralized tissue in vertebrates. Osteoclasts are actively motile cells that migrate along the surface of bone, and can bind to bone, secrete necessary acids and proteases, thereby causing the actual resorption of mineralized tissue of the bone. More specifically, osteoclasts are believed to exist in at least two physiological states, i.e. 35 the active/secretory state and the migratory or motile state. In the

secretory state, osteoclasts are flat, attach to the bone matrix via a tight attachment, i.e. sealing, zone, become highly polarized, form a ruffled border, and secrete lysosomal enzymes and protons to resorb bone. The adhesion of osteoclasts to bone surfaces is an important initial step in
5 bone resorption. In the migratory or motile state, the osteoclasts migrate across bone matrix and do not take part in resorption until they again attach to bone. Kinases such as Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase are believed to be involved in the regulation of osteoclast function. Therefore, by targeting these kinases,
10 osteoclast mediated disease states, for example osteoporosis, can be treated.

In the present invention, it was unexpectedly discovered that certain compounds that are known to modify osteoclast function activate stress responsive kinases. These activating compounds are the
15 bisphosphonate alendronate, the snake venom disintegrin echistatin, anti β 3 integrin monoclonal antibody (commercially available from Pharmingen, San Diego, CA), the naturally occurring polypeptide calcitonin, and the echistatin RGD (i.e. arginine, glycine, aspartate) mimetics 3(S)-(2-(2-oxo-3(S)-[5,6,7,8-tetrahydro-[1,8]-naphthyridin-2-ylmethyl)-amino]-pyrrolidin-1-yl)-acetylamino)-4-quinolin-3-yl-butyric acid trihydrochloride (hereafter "RGD I") and (2-[N-(3,4,5,6-tetrahydropyrimidin-2-yl)amino]ethoxyphen-4-yl)carbonyl-2(S)-phenylsulfonamido-beta-alanine (hereafter "RGD II"). This unexpected finding led to the further discovery of providing methods of identifying
20 compounds which activate these kinases and the realization that other compounds so-identified would be useful for treating a variety of conditions or disease states mediated by these kinases.
25

It is therefore an object of the present invention to provide methods for identifying compounds which modulate a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase and mixtures thereof.

It is another object of the present invention to provide methods of activating in mammalian cells a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase and mixtures thereof.

It is another object of the present invention to provide methods of eliciting an activating effect in a mammal of a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and mixtures thereof.

5 It is another object of the present invention to provide methods of treating or preventing a disease or condition in a mammal by modulating, for example activating, a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and mixtures thereof.

10 It is another object of the present invention to provide compounds that are useful for modulating, for example activating, a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and mixtures thereof.

15 It is another object of the present invention to provide pharmaceutical compositions comprising these compounds.

These and other objects will become readily apparent from the detailed description which follows.

20 SUMMARY OF THE INVENTION

The present invention relates to a method for identifying compounds which modulate a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and combinations thereof, comprising:

25 a). contacting a putative activity-modifying compound with a cell culture selected from the group consisting of an osteoclastogenesis cell culture, purified osteoclasts, partially-purified osteoclasts, unpurified osteoclasts, purified pre-osteoclasts, partially-purified pre-osteoclasts, unpurified pre-osteoclasts, purified osteoclast-like cells, partially-purified osteoclast-like cells, unpurified osteoclast-like cells, and mixtures thereof; and

30 b). determining the kinase activity of said cell culture and comparing said kinase activity with a cell culture not contacted with said putative activity-modifying compound.

In further embodiments, the present invention relates to a method of activating or otherwise modulating in a mammalian cell a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and combinations thereof, comprising contacting said cell with an activating or otherwise modulating amount of a compound identified according to the methods of the present invention.

5 In further embodiments, the present invention relates to a method of eliciting in a mammal an activating or otherwise modulating effect of a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and combinations thereof, comprising administering to said mammal an activating amount of a compound identified according to the methods of the present invention.

10 15 In further embodiments, the present invention relates to a method of treating or preventing a disease or condition in a mammal that is mediated by a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and combinations thereof, comprising administering to said mammal a pharmaceutically effective amount of a compound identified according to the methods of the present invention.

20 25 In further embodiments, the present invention relates to compounds useful for activating or otherwise modulating a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and combinations thereof.

In further embodiments, the present invention relates to pharmaceutical compositions comprising these compounds.

30 BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the time course for activation of a kinase responsive to stress by anti $\beta 3$ integrin monoclonal antibody (mAb) and by echistatin. Preosteoclasts (POCs) from bone marrow and osteoblast (MB 1.8) cocultures were isolated as collagenase-insensitive, EDTA-sensitive cells (80-90% purity). Approximately 20 hours after

purification, the cells were treated with anti $\beta 3$ integrin (mAb) (2 μ g/ml) (top Figure 1A) or echistatin (30 nM) (bottom Figure 1B) for the indicated times. Kinase activities were determined using an in-gel kinase assay method employing myelin basic protein as a substrate. An
5 approximately 60 kDa kinase doublet is strongly activated by both the anti $\beta 3$ integrin mAb and by echistatin. A signal is also observed for a kinase of approximately 50 kDa, which is also activated by these treatments.

FIG. 2 shows a dose response for activation of 50-60 kDa kinases by echistatin. Preosteoclasts from bone marrow and osteoblast
10 (MB 1.8) cocultures were isolated as collagenase-insensitive, EDTA-sensitive cells (80-90% purity). Approximately 20 hr after purification, cells were treated with varying concentrations of echistatin as indicated. Standard (i.e. overnight) film exposure (left panel Figure 2A) shows activation of an approximately 60 kDa kinase doublet at concentrations \geq
15 10 nM with maximal activities at about 30 nM. Extended (i.e. about 3-4 days) exposure (right panel Figure 2B) shows activation of the approximately 50 kDa kinase at the 30 and 100 nM doses.

FIG. 3 shows the identification of the approximately 60 kDa kinase doublet as the two isoforms of the Mammalian Sterile 20-like (Mst) kinase. Preosteoclasts from bone marrow and osteoblast (MB 1.8) cocultures were isolated as collagenase-insensitive, EDTA-sensitive cells (80-90% purity). Approximately 20 hr after purification, cells were treated with echistatin as indicated (a plus "+" sign indicating treatment and a minus "-" sign indicating no treatment). Cell lysates were exposed
20 to treatment with a cocktail of commercially available anti Mst and Krs antibodies and Protein A-agarose beads to immunodeplete Mst from the samples. Control depletions without antibodies failed to diminish the 60 kDa kinase doublet (lanes 1 and 2) while treatment with the Mst/Krs antibody cocktail eliminated >90% of these activities (lanes 3 and 4).
25 Corresponding activities associated with the Protein A-agarose beads showed that kinases were immunoprecipitated when the antibody cocktail was present in the mixture (lanes 7 and 8) and not when the antibody cocktail was absent (lanes 5 and 6).

FIG. 4 shows activation of Mst 1, Mst 2, and 50 kDa Kinase by the echistatin RGD mimetics RGD I (10 nM) and RGD II (10 μ M). Preosteoclasts from bone marrow and osteoblast (MB 1.8) cocultures were isolated as collagenase-insensitive, EDTA-sensitive cells (80-90% purity). Approximately 20 hr after purification, cells were treated with echistatin or the echistatin RGD mimetics. Timecourse (treatment and sampling at the indicated times) using echistatin to treat the cells shows maximal activity after 20 min treatment (left panel, Figure 4A). The right panel, Figure 4B shows that the echistatin RGD mimetics RGD I and RGD II are also effective in activating Mst 1, Mst 2, and 50 kDa kinase.

FIG. 5 shows that $\alpha v\beta 3$ integrin expression in HEK 293 cells (human embryonic kidney 293 cells ATCC CRL 1573 sold by American Type Culture Collection) reduces basal kinase activities of Mst 1, Mst 2, and 50 kDa kinase. This effect is reversed by treatment with echistatin and RGD II, but not by a mutant echistatin, whose Arg-Gly-Asp sequence has been changed to Ala-Gly-Asp (Ala²⁴ Echistatin). HEK 293 transflectants expressing human $\alpha v\beta 3$ integrin were treated with echistatin (30 nM, Figure 5A) or RGD II (10 μ M, Figure 5B) for 0 min (lane 1), 10 min (lane 2) 20 min (lane 3), 30 min (lane 4) and 60 min (lane 5). These treatments resulted in an increase in Mst 1 (ca. 59 kDa), Mst 2 (ca. 60 kDa), and 50 kDa Kinase activities. Direct comparison $\alpha v\beta 3$ integrin transfected HEK 293 cells (Figure 5D, lane 1) to Parental HEK 293 cells (Figure 5D, lane 2) shows that overexpression of $\alpha v\beta 3$ integrin reduces basal activities of Mst 1, Mst 2, and 50 kDa Kinase. Maximal activities observed in Figures 5A and 5B, which are generated in response to treatments with echistatin or RGD II, are comparable to basal activities observed in the Parental HEK 293 control cells (Figure 5D, lane 2, Figure 5E, lane 1, and Figure 5F, lane 1). Unlike in the transfected cells, treatment of Parental HEK 293 cells with echistatin (Figure 5D) for 10 min (lane 3), 20 min (lane 4), 30 min (lane 5), or 60 min (lane 6) had no significant effect on activities of Mst 1, Mst 2, and 50 kDa Kinase. Similar results were obtained when RGD II was used instead of echistatin (Figure 5E) for 10 min (lane 2), 20 min (lane 3), 30 min (lane 4)

and 60 min (lane 5). Neither transfected HEK 293 cells (figure 5C) nor Parental HEK 293 cells (Figure 5F) responded to Ala²⁴ echistatin (30 nM) for 0 min (lane 1), 10 min (lane 2), 30 min (lane 3), and 60 min (lane 4).

FIG. 6 shows the preferential response of 34 kDa Kinase to 5 alendronate monosodium trihydrate. Osteoclast like cells were treated with alendronate monosodium trihydrate, etidronate disodium, or tiludronate disodium at concentrations of 30 μ M and 100 μ M for about 17 hours. Kinase activities were analyzed using the in-gel kinase assays. Autoradiographs were developed after film exposure for several days.

10 These data show the activation of a 34 kDa Kinase by alendronate at either 30 μ M (lane 3) or 100 μ M (lane 4). Equivalent doses of etidronate disodium (lanes 5 and 6) or tiludronate disodium (lanes 7 and 8) failed to activate this kinase and were similar to no treatment controls (lanes 1 and 2).

15 FIG. 7 shows the effect of 10 μ M geranyl geraniol on the activation of osteoclast like cells with 30 μ M alendronate monosodium trihydrate. Lane 1 shows a no treatment control. Lane 2 shows the activation of a 34 kDa Kinase by alendronate. Lane 3 shows that geranyl geraniol negates this activation. Lane 4 is a treatment control with only 20 geranyl geraniol.

25 FIG. 8 shows the effect of 10, 15, and 60 μ M alendronate monosodium trihydrate, 10 μ M geranylgeraniol, 1 mM mevalonate, the combination of 10, 15, and 60 μ M alendronate monosodium trihydrate and 10 μ M geranylgeraniol, and the combination of 10, 15, and 60 μ M alendronate monosodium trihydrate and 1 mM mevalonate on osteoclast like cells in terms of the formation of cells larger than 300 μ m (as measured in one dimension). The compounds were dosed both 5 and 6 days after establishing the culture, and the formation of 300 μ m cells were counted using an inverted microscope with a 10X objective and 30 quantitated versus a no treatment control.

FIG. 9 shows the preferential response of 34 kDa Kinase to alendronate monosodium trihydrate and the inability of alendronate monosodium trihydrate to activate the 34 kDa Kinase when used to treat osteoclasts in the presence of the caspase inhibitor Z-Val-Ala-Asp(OMe)-

CH₂F (hereafter Z-VAD-FMK). Osteoclast-like cells were treated with alendronate monosodium trihydrate at concentrations of 10 µM and 30 µM for about 17 hours. Kinase activities were analyzed using in-gel kinase assays. Autoradiographs were developed after film exposure for several days. These data show the activation of a 34 kDa Kinase by alendronate at either 10 µM (lane 2) or 30 µM (lane 3). Alendronate does not activate the 34 kDa Kinase when coincubated in the presence of Z-VAD-FMK (lane 8). Higher doses of etidronate disodium, i.e. 100 µM (lane 4) and 300 µM (lane 5), or tiludronate disodium, i.e. 100 µM (lane 6) and 300 µM (lane 7), failed to activate this kinase and were similar to no treatment control (lane 1). See J.D. Graves et al., "Caspase-mediated activation and induction of apoptosis by the mammalian Ste2-like kinase Mst1", The EMBO Journal, vol. 17, no. 8, pp. 2224-2234 (1998), which is incorporated by reference herein in its entirety.

FIG. 10 shows identification of 34 kDa kinase activated by alendronate monosodium trihydrate (30 µM) as an amino-terminal cleavage product of Mst. Osteoclast-like cells were isolated and treated with alendronate monosodium trihydrate. Cell lysates were exposed to treatment with a cocktail of commercially available anti-Mst and anti-Krs antibodies that recognize the amino-terminus of Mst kinase and Protein A-agarose beads to immunoprecipitate Mst from the samples. These were analyzed using the in-gel kinase assay. These data demonstrate that the anti-Mst and anti-Krs antibody cocktail immunoprecipitates the full length 59 and 60 kDa Mst Kinases as well as the 34 kDa Mst Kinase cleavage product in untreated osteoclasts (lane 2) and alendronate-treated osteoclasts (lane 3). No kinase activities are immunoprecipitated in the absence of anti-Mst and anti-Krs antibodies (lane 1). These data also demonstrate that the 34 kDa Kinase immunoprecipitated by the anti-Mst and anti-Krs antibody cocktail is activated by alendronate monosodium trihydrate (lane 3).

DETAILED DESCRIPTION OF THE INVENTION

Several methods of inhibiting osteoclast activity in mammals, and in particular for treating osteoporosis, are known.

These methods include treatment with bisphosphonates [for example alendronate (especially alendronate monosodium trihydrate), cimadronate, clodronate, tiludronate, etidronate, ibandronate, neridronate, olpandronate, risedronate, piridronate, pamidronate,

- 5 zolendronate, pharmaceutically acceptable salts thereof, and mixtures thereof], hormones such as estrogen, or certain peptides such as calcitonin.

In the present invention, an approach has been employed using in-gel kinase assays to identify kinases whose activities change in
10 the osteoclast system during treatment with these and other agents.

- Identification of the two isoforms of the Mammalian Sterile 20-like (Mst) kinases, Mst 1 and Mst 2, and of the 34 kDa Kinase, the 50 kDa Kinase, and the 130 kDa Kinase, provided the first readout of signal transduction and stress response pathways that respond to these drugs. This initial
15 work led to the unexpected discovery that it would be possible to identify compounds that are useful for activating these kinases and that such activator compounds would be useful for treating or preventing disease states mediated by such kinases.

In the present invention, the kinases that are activated, i.e.
20 Mst 1, Mst 2, the 34 kDa Kinase, the 50 kDa Kinase, and the 130 kDa Kinase are identified with in-gel kinase assays as in Example 2. Also, standard immunoprecipitation techniques have been employed in the further identification of Mst 1 and Mst 2. An aspect of this invention is an assay for the identification of compounds which modulate, preferably
25 activate, these kinases. This comprises the steps of contacting a putative activity-modifying compound with an osteoclastogenesis cell culture, and determining the kinase activity of the cell culture by comparing its kinase activity with a control cell culture, i.e. a cell culture that had not been contacted with the activity-modifying compound. A wide variety of
30 cell cultures known to one of ordinary skill in the art can be employed. Preferably, the cultures are selected from the group consisting of an osteoclastogenesis cell culture, purified osteoclasts, partially-purified osteoclasts, unpurified osteoclasts, purified pre-osteoclasts, partially-purified pre-osteoclasts, unpurified pre-osteoclasts, purified osteoclast-like cells, partially-purified osteoclast-like cells, unpurified osteoclasts-

like cells, and mixtures thereof. A preferred cell culture is one containing mammalian bone marrow cells. For example, purified, i.e. about 80-90% pre-osteoclast cells generated by co-culturing mouse bone marrow cells and mouse calvaria osteoblasts can be used. Alternatively, 5 similar cells derived from avian bone sources can be used.

A wide variety of techniques known to one of ordinary skill in the art can be employed for measuring kinase activity. A preferred methodology involves an in-gel kinase assay in which the samples are subjected to electrophoresis in the presence of an appropriate 10 immobilized kinase substrate and then measured for kinase activity against that substrate. This method provides a convenient technique for assaying multiple samples and rapidly quantitating the activity of the electrophoresed kinases.

Other techniques that can be used to quantitate kinase 15 activity include, but are not limited to, immunoprecipitation, chromatography, addition of selective inhibitors for retaining the activity of only the desired kinase, and use of selective substrates. In these assays, kinase activities are generally assayed in-solution rather than in-gel.

20 Methods for modulating a kinase responsive to stress in a mammalian cell

The present invention also relates to a method for modulating, e.g. activating, a kinase responsive to stress in a mammalian cell, wherein said kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 25 130 kDa Kinase, and combinations thereof. These methods comprise contacting said cell with an activating amount of a compound selected from the group consisting of an organic bisphosphonate, echistatin, anti β 3 integrin monoclonal antibody, calcitonin, 3(S)-(2-(2-oxo-3(S)-[5,6,7,8-tetrahydro-[1,8]-naphthyridin-2-ylmethyl)-amino]-pyrrolidin-1-yl)-30 acetylamino)-4-quinolin-3-yl-butyric acid trihydrochloride, (2-[N-(3,4,5,6-tetrahydropyrimidin-2-yl)amino]ethoxyphen-4-yl)carbonyl-2(S)-phenylsulfonamido-beta-alanine, and mixtures thereof.

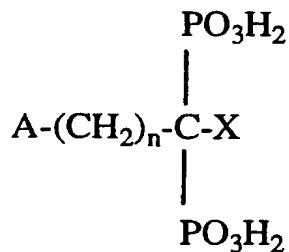
Without being limited by theory, it is believed that as a 35 subclass of the organic bisphosphonates, that the nitrogen containing

bisphosphonates act by inhibiting protein prenylation, most critically geranylgeranylation. Nitrogen containing bisphosphonates such as alendronate, risedronate, and pamidronate induce activation of the 34 kDa Mst kinase. Clodronate, and to a lesser extent, etidronate (both non-nitrogen containing bisphosphonates) also induce the 34 kDa Mst kinase. It is found that the mevalonate pathway is inhibited with lovastatin, thus blocking the genesis of isoprenyl and cholesterol precursors. Treatment with lovastain for 17-24 hours increases the 34 kDa Mst 1 activity, indicating that a block in protein prenylation or 5 cholesterol biosynthesis can cause Mst 1 cleavage. It is found that alendronate- and risedronate-induced Mst 1 cleavage is essentially completely blocked by geranylgeraniol, while farnesol and mevalonic acid lactone reduce cleavage by about 50%. Lovastatin effects are blocked by mevalonic acid or geranylgeraniol, but not by farnesol. Clodronate 10 effects are not blocked by geranylgeraniol, suggesting that clodroante does not act by inhibiting protein prenylation. These findings suggest that Mst kinase is part of a stress responsive pathway activated in purified osteoclasts by nitrogen containing and non-nitrogen containing bisphosphonates. Geranylgeranylation appears to play a critical role in 15 the mechanism of action of nitrogen containing bisphosphonates, but not in the mechanism of action of the non-nitrogen containing bisphosphonates. Mst 1 cleavage is mediated by caspases downstream of geranylgeranylated proteins, a group that includes small G-proteins that regulate apoptosis, the cytoskeleton, and vesicular trafficking. 20 However, even though nitrogen containing bisphosphonates may act via a different mechanism of action, these findings are in no way intended to limit the present invention to such bisphosphonates.

25

30 **Organic Bisphosphonates**

The methods and compositions of the present invention, can comprise a bisphosphonate active. The bisphosphonates of the present invention correspond to the chemical formula



wherein n is an integer from 0 to 7 and wherein A and X are independently selected from the group consisting of H, OH, halogen, 5 NH₂, SH, phenyl, C1-C30 alkyl, C3-C30 branched or cycloalkyl, C1-C30 substituted alkyl, C1-C10 alkyl substituted NH₂, C3-C10 branched or cycloalkyl substituted NH₂, C1-C10 dialkyl substituted NH₂, C3-C10 branched or cycloalkyl disubstituted NH₂, C1-C10 alkoxy, C1-C10 alkyl substituted thio, thiophenyl, halophenylthio, C1-C10 alkyl substituted phenyl, pyridyl, furanyl, pyrrolidinyl, imidazolyl, imidazopyridinyl, and 10 benzyl, such that both A and X are not selected from H or OH when n is 0; or A and X are taken together with the carbon atom or atoms to which they are attached to form a C3-C10 ring.

In the foregoing chemical formula, the alkyl groups can be 15 straight, branched, or cyclic, provided that sufficient atoms are selected for the chemical formula. The C1-C30 substituted alkyl can include a wide variety of substituents, nonlimiting examples which include those selected from the group consisting of phenyl, pyridyl, furanyl, pyrrolidinyl, imidazonyl, NH₂, C1-C10 alkyl or dialkyl substituted NH₂, 20 OH, SH, and C1-C10 alkoxy.

The foregoing chemical formula is also intended to encompass complex carbocyclic, aromatic and hetero atom structures for the A and/or X substituents, nonlimiting examples of which include naphthyl, quinolyl, isoquinolyl, adamantyl, and chlorophenylthio.

A non-limiting class of structures useful in the instant 25 invention are those in which A is selected from the group consisting of H, OH, and halogen, X is selected from the group consisting of C1-C30 alkyl, C1-C30 substituted alkyl, halogen, and C1-C10 alkyl or phenyl substituted thio, and n is 0.

A non-limiting subclass of structures useful in the instant invention are those in which A is selected from the group consisting of H, OH, and Cl, X is selected from the group consisting of C1-C30 alkyl, C1-C30 substituted alkyl, Cl, and chlorophenylthio, and n is 0.

5 A non-limiting example of the subclass of structures useful in the instant invention is when A is OH and X is a 3-aminopropyl moiety, and n is 0, so that the resulting compound is a 4-amino-1,-hydroxybutylidene-1,1-bisphosphonate, i.e. alendronate.

10 Pharmaceutically acceptable salts and derivatives of the bisphosphonates are also useful herein. Nonlimiting examples of salts include those selected from the group consisting alkali metal, alkaline metal, ammonium, and mono-, di, tri-, or tetra-C1-C30-alkyl-substituted ammonium. Preferred salts are those selected from the group consisting of sodium, potassium, calcium, magnesium, and 15 ammonium salts. Nonlimiting examples of derivatives include those selected from the group consisting of esters, hydrates, and amides.

15 It should be noted that the terms "bisphosphonate" and "bisphosphonates", as used herein in referring to the therapeutic agents of the present invention are meant to also encompass diphosphonates, 20 biphosphonic acids, and diphasphonic acids, as well as salts and derivatives of these materials. The use of a specific nomenclature in referring to the bisphosphonate or bisphosphonates is not meant to limit the scope of the present invention, unless specifically indicated. Because of the mixed nomenclature currently in use by those of ordinary skill in 25 the art, reference to a specific weight or percentage of a bisphosphonate compound in the present invention is on an acid active weight basis, unless indicated otherwise herein. For example, the phrase "about 5 mg of a bisphosphonate selected from the group consisting of alendronate, pharmaceutically acceptable salts thereof, and mixtures thereof, on an 30 alendronic acid active weight basis" means that the amount of the bisphosphonate compound selected is calculated based on 5 mg of alendronic acid. For other bisphosphonates, the amount of bisphosphonate is calculated based on the corresponding bisphosphonic acid.

Nonlimiting examples of bisphosphonates useful herein include the following:

Alendronic acid, 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid.

5 Alendronate (also known as alendronate sodium or alendronate monosodium trihydrate), 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid monosodium trihydrate.

10 Alendronic acid and alendronate are described in U.S. Patents 4,922,007, to Kieczykowski et al., issued May 1, 1990; 10 5,019,651, to Kieczykowski et al., issued May 28, 1991; 5,510,517, to Dauer et al., issued April 23, 1996; 5,648,491, to Dauer et al., issued July 15, 1997, all of which are incorporated by reference herein in their entirety.

15 Cycloheptylaminomethylene-1,1-bisphosphonic acid, YM 175, Yamanouchi (cimadronate), as described in U.S. Patent 4,970,335, to Isomura et al., issued November 13, 1990, which is incorporated by reference herein in its entirety.

20 1,1-dichloromethylene-1,1-diphosphonic acid (clodronic acid), and the disodium salt (clodronate, Procter and Gamble), are described in Belgium Patent 672,205 (1966) and *J. Org. Chem.* 32, 4111 (1967), both of which are incorporated by reference herein in their entirety.

25 1-hydroxy-3-(1-pyrrolidinyl)-propylidene-1,1-bisphosphonic acid (EB-1053).

30 1-hydroxyethane-1,1-diphosphonic acid (etidronic acid). 1-hydroxy-3-(N-methyl-N-pentylamino)propylidene-1,1-bisphosphonic acid, also known as BM-210955, Boehringer-Mannheim (ibandronate), is described in U.S. Patent No. 4,927,814, issued May 22, 1990, which is incorporated by reference herein in its entirety.

35 6-amino-1-hydroxyhexylidene-1,1-bisphosphonic acid (neridronate).

40 3-(dimethylamino)-1-hydroxypropylidene-1,1-bisphosphonic acid (olpadronate).

3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid (pamidronate).

5 [2-(2-pyridinyl)ethylidene]-1,1-bisphosphonic acid (piridronate) is described in U.S. Patent No. 4,761,406, which is incorporated by reference in its entirety.

1-hydroxy-2-(3-pyridinyl)-ethylidene-1,1-bisphosphonic acid (risedronate).

10 (4-chlorophenyl)thiomethane-1,1-disphosphonic acid (tiludronate) as described in U.S. Patent 4,876,248, to Breliere et al., October 24, 1989, which is incorporated by reference herein in its entirety.

15 1-hydroxy-2-(1^H-imidazol-1-yl)ethylidene-1,1-bisphosphonic acid (zolendronate).

20 A non-limiting class of bisphosphonates useful in the instant invention are selected from the group consisting of alendronate, cimadronate, clodronate, tiludronate, etidronate, ibandronate, neridronate, olpandronate, risedronate, piridronate, pamidronate, zolendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

25 A non-limiting subclass of the above-mentioned class in the instant case is selected from the group consisting of alendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

A non-limiting example of the subclass is alendronate monosodium trihydrate.

30

The following Examples are presented to better illustrate the invention.

EXAMPLE 1

Preparation of Preosteoclasts and Osteoclast-like cells from Bone Marrow/Calvaria Cell Cocultures

The following procedure is a modification of Wesolowski et al. (1995, *Exp. Cell Res.* 219: 679-686), which is incorporated by reference herein in its entirety. Cocultures are made by adding marrow cells from

6-week old mouse tibia and femur onto a monolayer of MB 1.8 mouse calvaria osteoblasts that are preseeded into culture dishes in α -MEM (minimal essential medium) containing fetal calf serum (10%) and 1,25 Dihydroxy Vitamin D3 (10 nM). Cocultures are maintained, with media changes every 48 hours, for a period of 6 days. Non-osteoclastic cells are removed by treatment of cocultures with Collagenase I (1 mg/ml in Phosphate Buffered Saline [PBS]). Collagenase-sensitive cells are removed by washing with PBS. Preosteoclasts are lifted from the dish using EDTA (0.2 g/L in PBS) and transferred into culture dishes and maintained in α -MEM containing fetal calf serum (10%), 1,25 Dihydroxy Vitamin D3 (10 nM), and m-Colony Stimulating Factor (5 ng/ml). Osteoclast-like cells (collagenase-insensitive, EDTA-insensitive cells) that remain attached to the original coculture dishes are maintained in α -MEM containing fetal calf serum (10%), 1,25 Dihydroxy Vitamin D3 (10 nM), and m-Colony Stimulating Factor (5 ng/ml).

EXAMPLE 2

Method to Examine Activity Change in Mst1, Mst2, and 50 kDa Kinase in Response to Treatment of Osteoclast-Like Cells with Inhibitors.

Preosteoclasts are generated and purified as described in Example 1. After cells have recovered 1-2 hr at 37°C, cells are treated with echistatin (typically 30 nM, although a range of 1 pM to 1 μM can be used). Untreated cells are included in the analyses and act as a negative control for kinase activities. Cells are then harvested and lysed in a HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethansulfonic acid) or Tris buffer containing the following: β-glycerophosphate (50 mM); Na₃VO₄ (1mM); NaF (1mM); Microcystin LR (1 μM); leupeptin (10 μg/ml); aprotinin (10 μg/ml); phenylmethyl sulfonylfluoride (1 mM). Protein concentrations are determined for each lysate and 5-20 μg are loaded into each lane of a SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel containing Myelin Basic Protein, or another kinase substrate, which has been polymerized into the gel at a concentration between 50-400 μg/ml. Molecular weight standards are also loaded into one or more lanes of the gels. In-gel kinase assays are run according to a standard procedure based on Kameshita and Fujisawa, 1989 (Anal. Biochem. 183:139-143) and of Gotoh et al., 1990 (Eur. J. Biochem. 193: 661-669), both references being incorporated by reference herein in their entirety. The proteins are electrophoresed in the above gels. The gels are then successively soaked in 50 mM HEPES, pH 7.6; 5 mM 2-mercaptoethanol and each of the following (for each wash): (a) 20% isopropanol; (b) no additions; (c) urea (6 M); (d) Urea (3 M); (e) Urea (0.75 M); and Tween 20 (0.05% vol:vol). Kinase reactions are then run by first soaking the gels in 20 mM HEPES, pH 7.6; 20 mM MgCl₂; 2 mM DTT and then in the same buffer containing 0.02 M ATP (non-radioactive) with ca. 1000 cpm/pmol ³²P-γ-ATP. The gels are then washed six times with 5% trichloroacetic acid and 1% pyrophosphate. The gels are then stained with Coomassie brilliant blue dye (0.125%) in 50% methanol, 10% acetic acid; destained with 30% methanol, 10% acetic acid; soaked in 2% glycerol; and dried using a gel dryer. The gels are then exposed to autoradiography film for times ranging from several hours to weeks.

The bands observed in the autoradiographs representing the gels reflect kinase activities. Mst 1 (apparent molecular weight about 59 kDa), Mst 2 (apparent molecular weight about 60 kDa), and 50 kDa kinase are observed and identified by their migration as compared to the migration 5 of molecular weight standards. The band intensities on the autoradiography film are quantitated by densitometry and comparisons between bands from untreated controls and bands from echistatin-treated cells provide the basis for the analyses.

A similar approach is used for alendronate, calcitonin, anti 10 β 3 integrin monoclonal antibody, RGD I, and RGD II.

A similar approach is also used to assay for the 34 kDa Kinase and the 130 kDa Kinase.

EXAMPLE 3

General Method for Identifying Compounds which Activate Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase in Osteoclast-Like Cells.

- 5 Osteoclast-like cells are generated as described in Example 1. The compound or compounds to be evaluated are added at the desired concentration to the osteoclast-like cells for any of various times (1 minute to several hours). Cell lysates are made using standard techniques and in-gel kinase assays are performed as described in
10 Example 2. Compounds that inhibit osteoclast function are identified by their ability to stimulate Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase and/or 130 kDa Kinase activities.

15

WHAT IS CLAIMED IS:

1. A method for identifying compounds which activate a kinase responsive to stress comprising:

5 a). contacting a putative activity-modifying compound with a cell culture; and

b). determining the kinase activity of said cell culture and comparing said kinase activity with a cell culture not contacted with said putative activity-modifying compound.

10

2. A method according to Claim 1 wherein said kinase responsive to stress is selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase and combinations thereof.

15

3. A method according to Claim 2 wherein said cell culture is selected from the group consisting of an osteoclastogenesis cell culture, purified osteoclasts, partially-purified osteoclasts, unpurified osteoclasts, purified pre-osteoclasts, partially-purified pre-osteoclasts, 20 unpurified pre-osteoclasts, purified osteoclast-like cells, partially-purified osteoclast-like cells, unpurified osteoclasts-like cells, and mixtures thereof.

25

4. A method according to Claim 3 wherein said kinase activity is determined using an in-gel kinase assay.

5. A method of activating a kinase responsive to stress in a mammalian cell comprising contacting said cell with an activating amount of a compound identified according to Claim 1.

30

6. A method of eliciting an activating effect of a kinase responsive to stress in a mammal comprising administering to said mammal an activating amount of a compound identified according to Claim 1.

35

7. A method of treating or preventing a disease or condition in a mammal that is mediated by a kinase responsive to stress comprising administering to said mammal a pharmaceutically effective amount of a compound identified according to Claim 1.

5

8. A method of treating or preventing osteoporosis, vascular restenosis, diabetic retinopathy, macular degeneration, angiogenesis, atherosclerosis, inflammation, or tumor growth in a mammal comprising administering to said mammal a pharmaceutically effective amount of a compound identified according to 10 Claim 1.

9. A compound identified according to Claim 1.

15

10. A pharmaceutical composition comprising the compound of Claim 9.

20

11. A method of activating in a mammalian cell a kinase responsive to stress selected from the group consisting of Mst 1, Mst 2, 34 kDa Kinase, 50 kDa Kinase, and 130 kDa Kinase, and combinations thereof, comprising contacting said cell with an activating amount of a compound selected from the group consisting of an organic bisphosphonate, echistatin, anti β 3 integrin monoclonal antibody, calcitonin, 3(S)-(2-(2-oxo-3(S)-[5,6,7,8-tetrahydro-[1,8]-naphthyridin-2-ylmethyl)-amino]-pyrrolidin-1-yl)-acetyl amino)-4-quinolin-3-yl-butyric acid trihydrochloride, (2-[N-(3,4,5,6-tetrahydropyrimidin-2-yl)amino]ethoxyphen-4-yl)carbonyl-2(S)-phenylsulfonamido-beta-alanine, and mixtures thereof.

25

30
35

12. A method according to Claim 11 wherein said organic bisphoshonate is selected from the group consisting of alendronate, cimadronate, clodronate, tiludronate, etidronate, ibandronate, neridronate, olpandronate, risedronate, piridronate, pamidronate, zolendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

13. A method according to Claim 11 wherein said organic bisphosphonate is alendronate and pharmaceutically acceptable salts thereof.

5

14. A method according to Claim 11 wherein said organic bisphosphonate is alendronate monosodium trihydrate.

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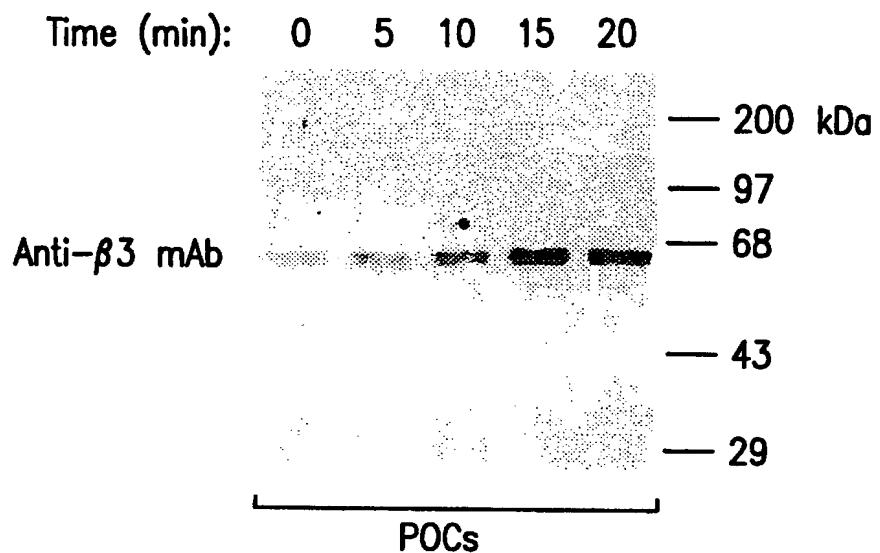


FIG. 1A

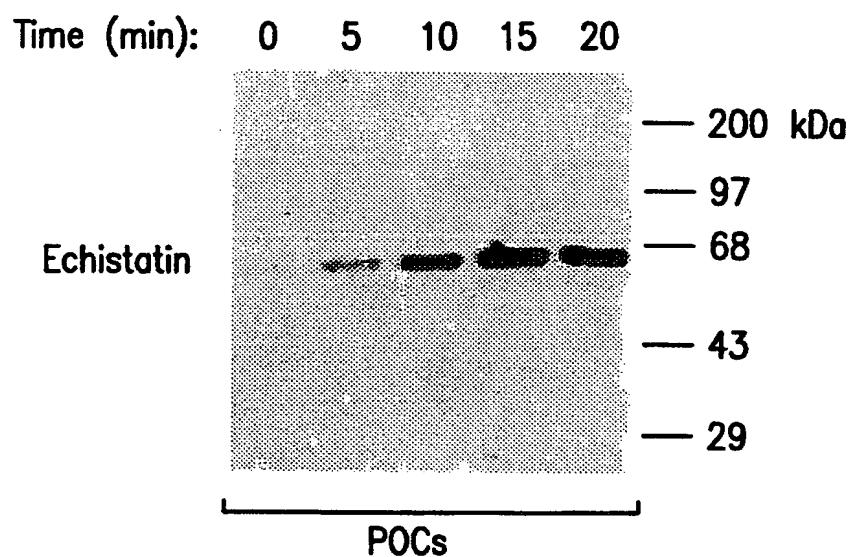


FIG. 1B

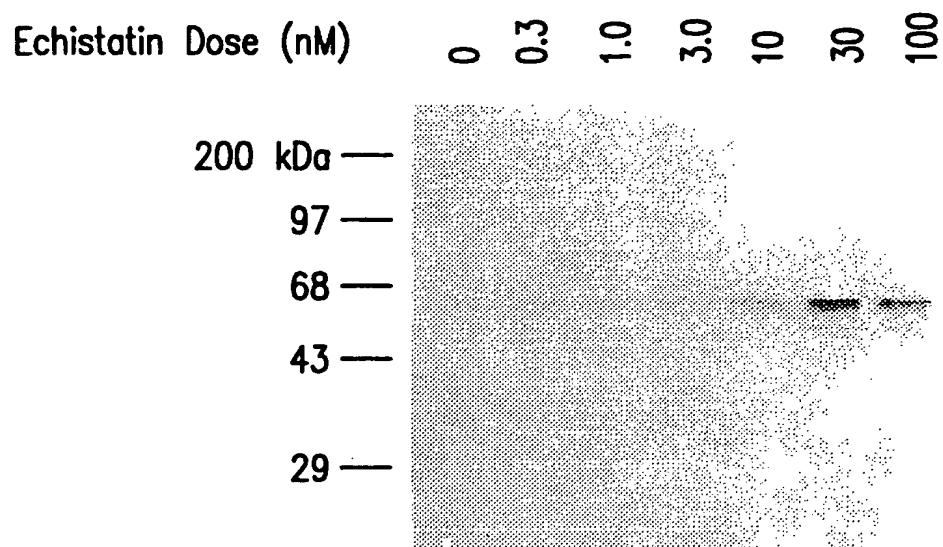


FIG. 2A

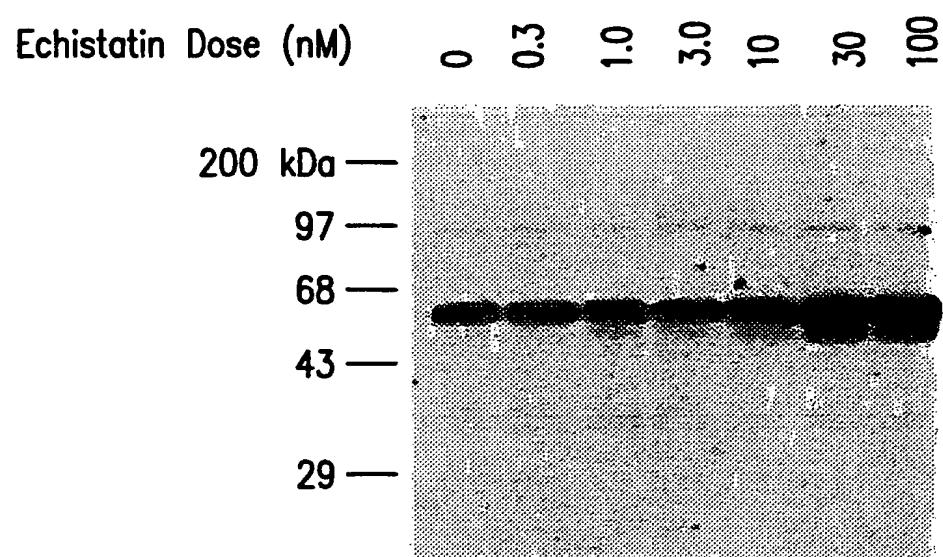


FIG. 2B

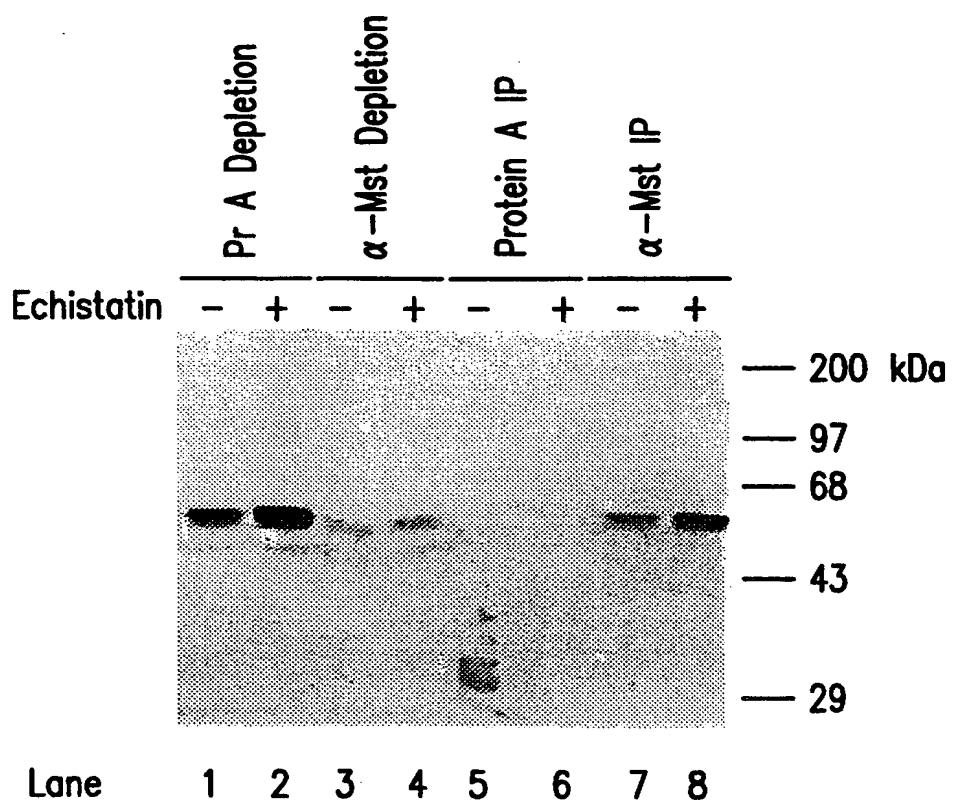


FIG.3

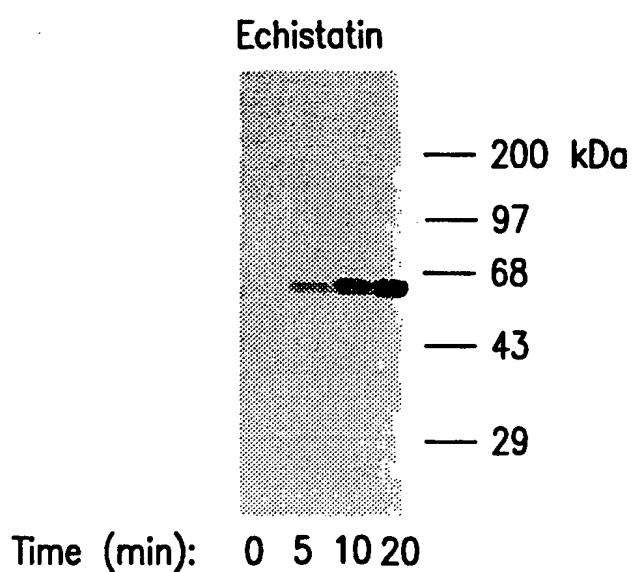


FIG.4A

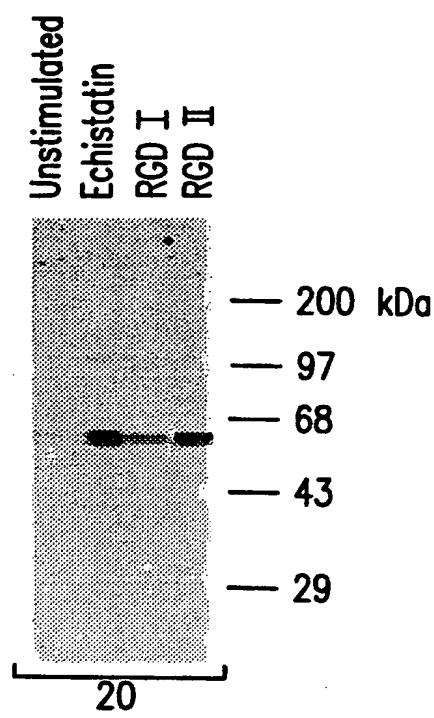


FIG.4B

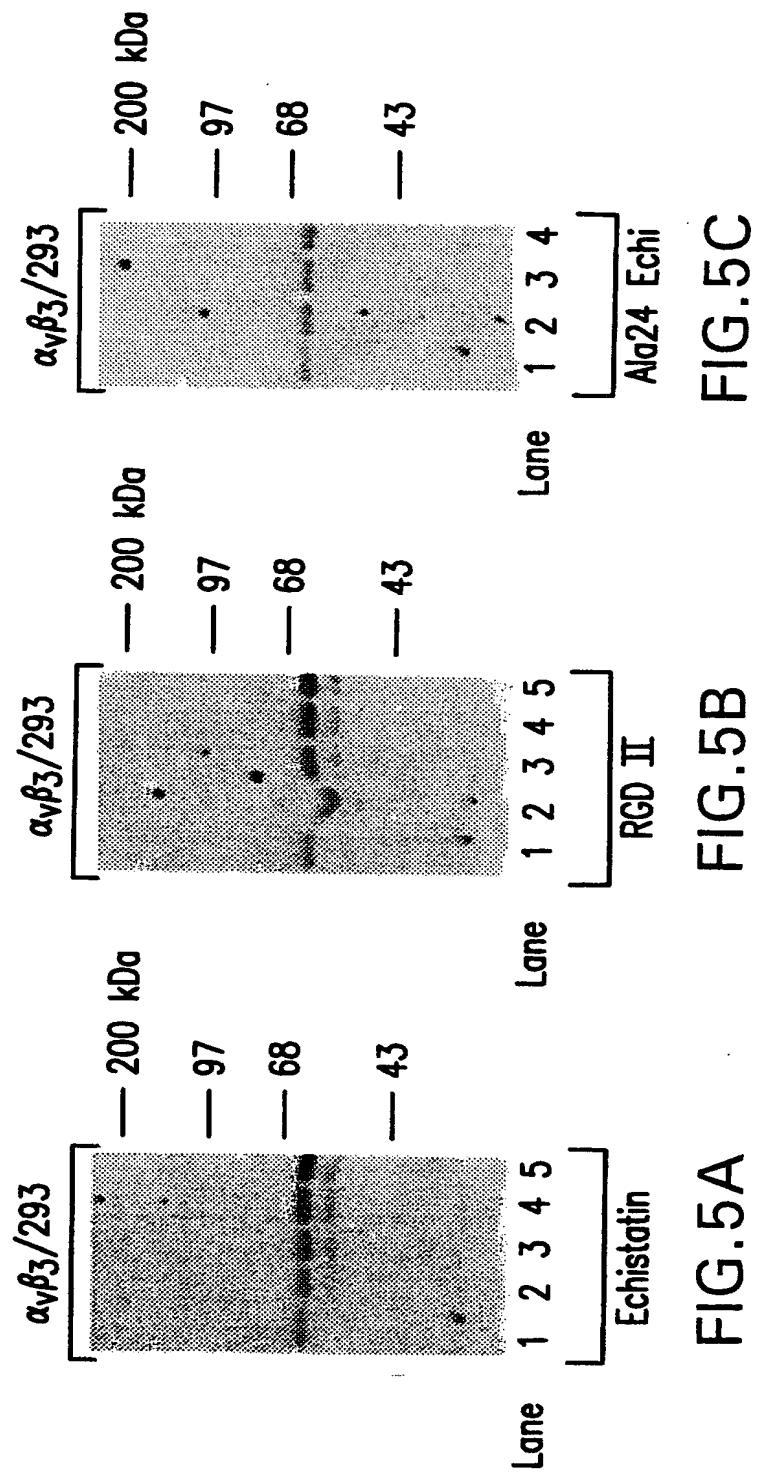


FIG. 5A

FIG. 5B

FIG. 5C

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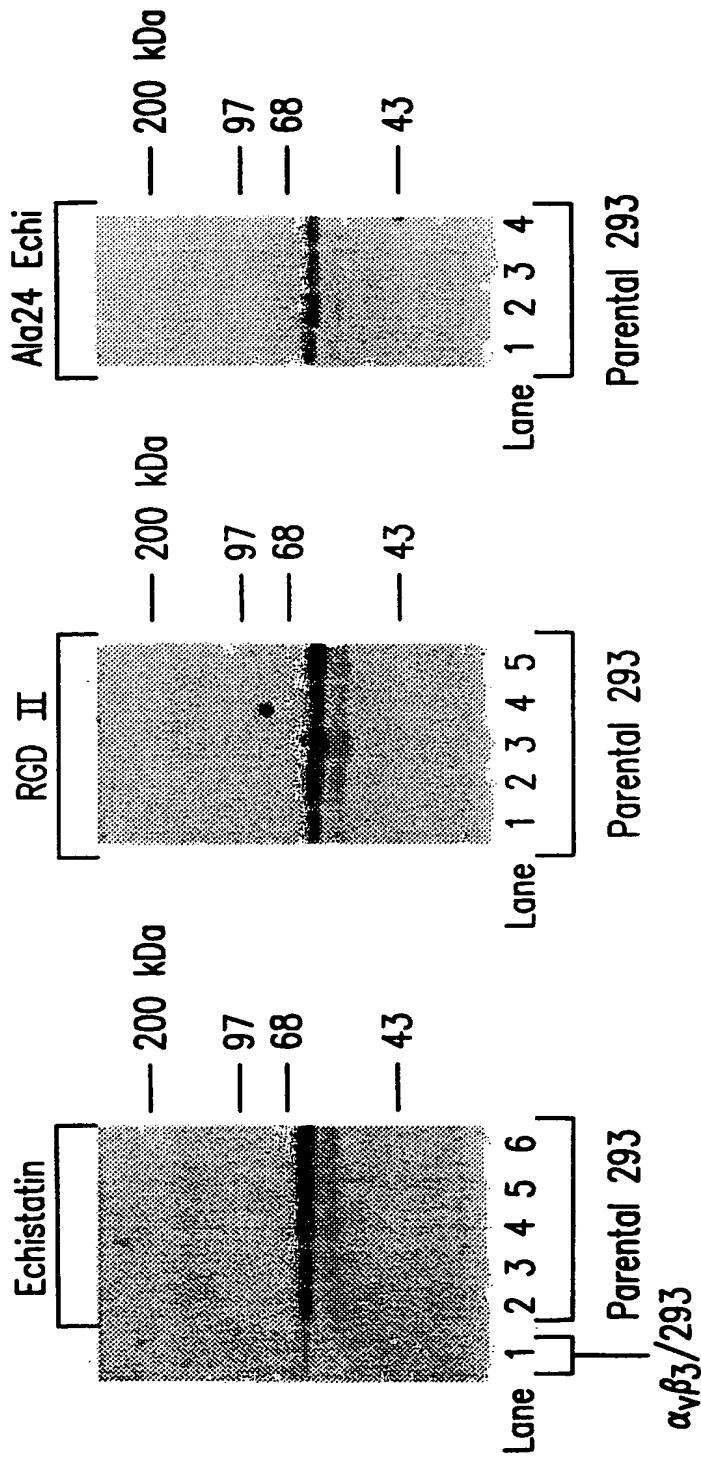


FIG.5D

FIG.5E

FIG.5F

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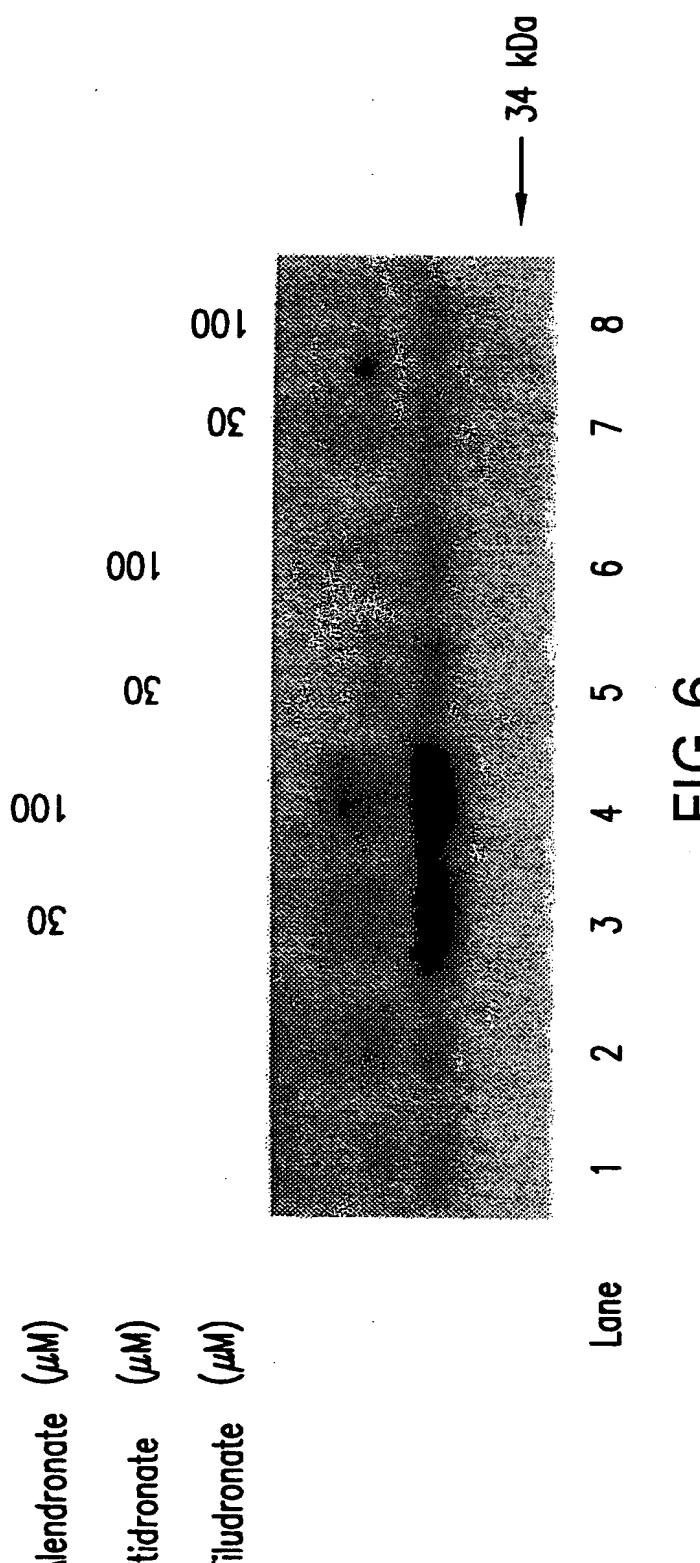


FIG. 6

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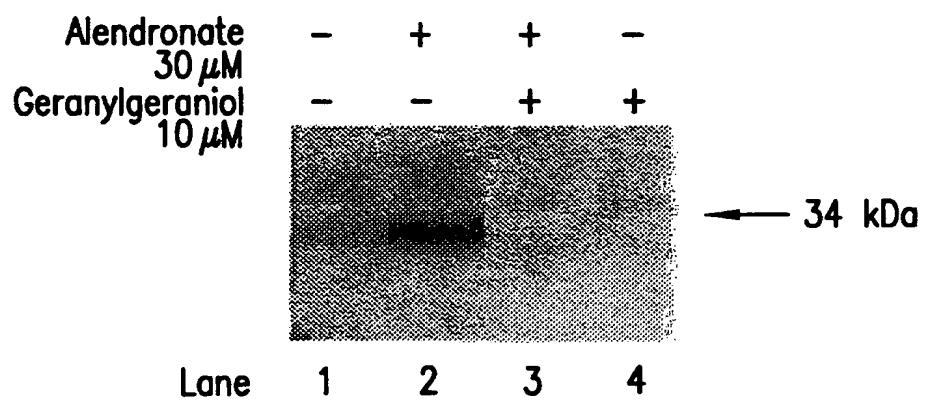
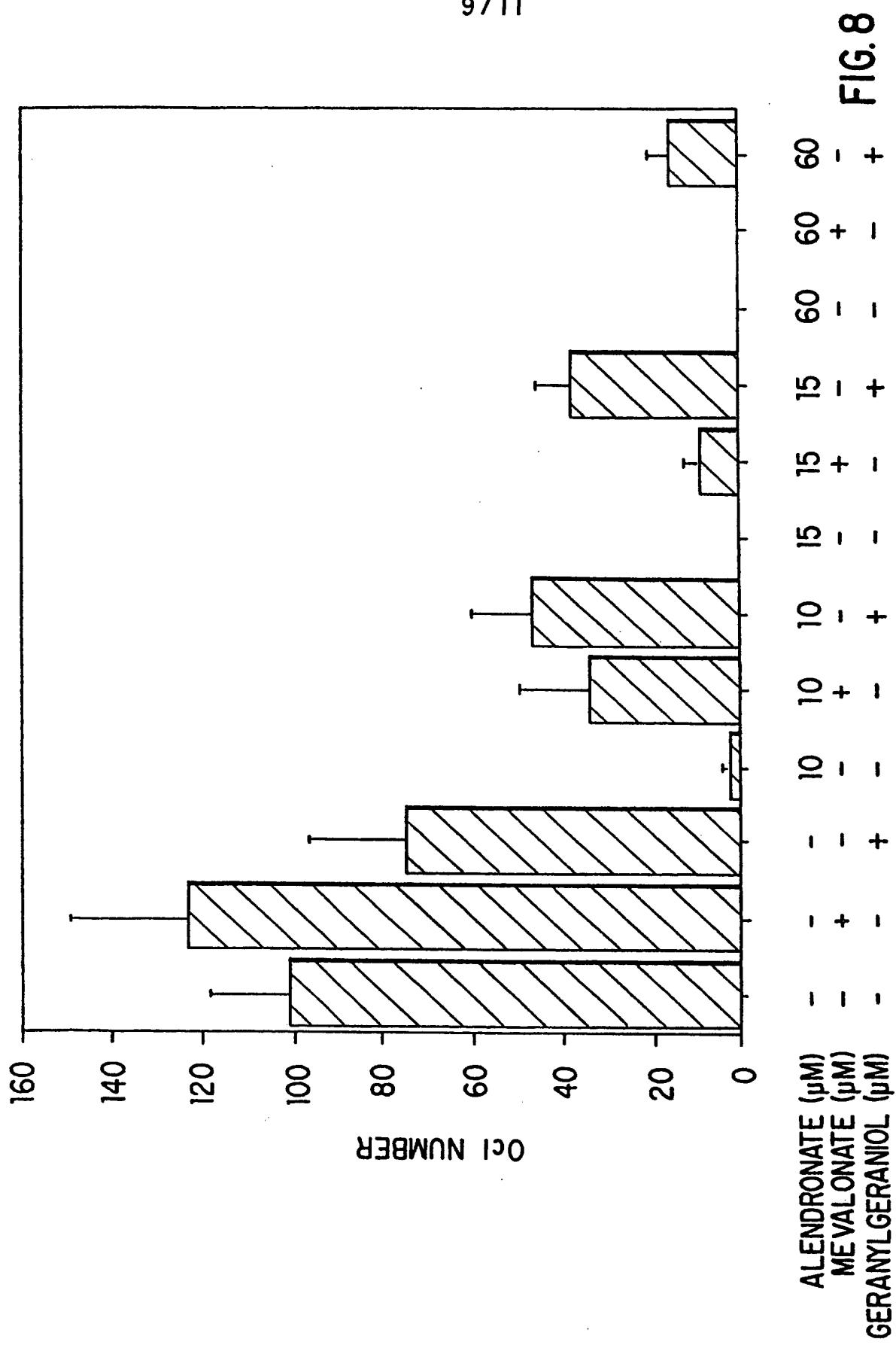


FIG. 7

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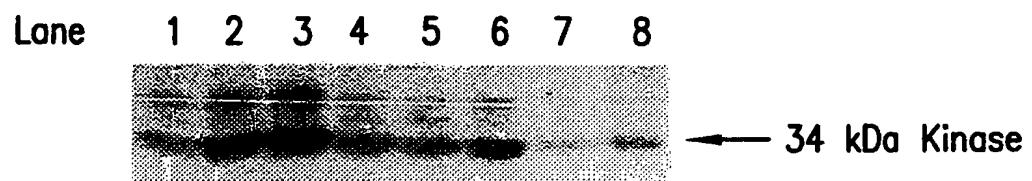


FIG.9

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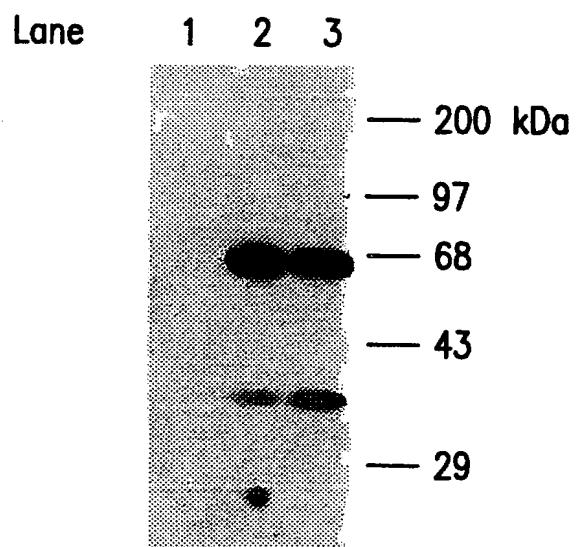


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02065

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/58; C12N 9/12; A01N 25/00

US CL : 435/15, 194; 514/789

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/15, 194; 514/789

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, BIOTECHDS, SCISEARCH, LIFESCI, HCAPLUS, NTIS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-------------|--|-----------------------------|
| X | SCHINKMANN et al. Cloning and characterization of a Human STE20-like protein kinase with unusual cofactor requirements. J. Biol. Chem. 07 November 1997, Vol.272, No.45, pages 28695-28703, see the entire article specially Table I, and pages 28700-28702. | 1, 5-10 |
| X,P | US 5,830,699 A (FORCE et al) 03 November 1998 (11/3/98), see the entire document , specially Table 1. | 1, 5-10 |
| X - Y | CREASY et al. Cloning and characterization of a Human Protein Kinase with homology to Ste20. J. Biol. Chem. 15 September 1995, Vol.270, No.37, pages 21695-21700, see the entire article, specially Fig 6 and page 21699. | 1-2, 5-10 ----- 11-14 |

Further documents are listed in the continuation of Box C. See patent family annex.

| | | |
|---|-----|--|
| * Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *B* earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | | |
| *P* document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search

14 APRIL 1999

Date of mailing of the international search report

20 MAY 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02065

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | SCHMIDT et al. Protein-tyrosine phosphatase activity regulates osteoclast formation and function: inhibition by alendronate Proc. Natl. Acad. Sci., U.S.A. 11 April 1996, Vol.93, pages 3068-3073; see the entire article. | 11-14 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/02065

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02065

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

THIS INTERNATIONAL SEARCHING AUTHORITY HAS FOUND 2 INVENTIONS CLAIMED IN THE INTERNATIONAL APPLICATION COVERED BY THE CLAIMS INDICATED BELOW:

GROUP I. CLAIMS 1-4, DRAWN TO A METHOD OF IDENTIFYING COMPOUNDS WHICH ACTIVATE A STRESS RESPONSIVE KINASE IN A MAMMALIAN CELL FROM THE GROUP CONSISTING OF Mst 1, Mst 2, 34 kDa KINASE, 50 kDa KINASE AND 130 kDa KINASE AND THE COMBINATIONS THEREOF.

GROUP II. CLAIMS 5-14, DRAWN TO A METHOD OF ACTIVATING A KINASE RESPONSIVE TO STRESS IN A MAMMALIAN CELL WITH AN ACTIVATING COMPOUND, THE ACTIVATING COMPOUND AND A PHARMACEUTICAL COMPOSITION COMPRISING THE ACTIVATING COMPOUND.

THE INVENTIONS LISTED AS GROUPS I-II DO NOT RELATE TO A SINGLE INVENTIVE CONCEPT BECAUSE THEY ARE CONSIDERED TO BE TWO DIFFERENT CATEGORIES OF INVENTION AND ARE NOT DRAWN TO COMBINATIONS OF CATEGORIES (I.E. CATEGORIES 1-5), SPECIFIED IN 37 CFR SECTION 1.475(B).